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Morphological Evidence for a Chitin-Like Glycoprotein in Penaeid Hatching Envelopes

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Abstract. When chitin hydrolytic enzymes (chitinase and *N*-acetylglucosaminidase) were used as treatment during formation of the hatching envelope (HE) of the penaeid shrimp *Sicyonia ingentis*, results indicated the presence of carbohydrate moieties in the envelope. Eggs exposed to these enzymes had abnormal HEs that might elevate and often collapsed. The finding that chitin synthase inhibitors (tunicamycin, nikkomycin Z, and polyoxin D) also interfered with normal HE formation is further evidence for a carbohydrate component. The application of these synthase inhibitors resulted in more fragile envelopes that elevated and collapsed or were easily lost during processing. Similar results were seen in the absence of divalent ions (magnesium and calcium) considered critical for normal chitin formation. This morphological evidence is indicative of a chitin-like, linked carbohydrate in the HE of *Sicyonia ingentis*.

Introduction

Oocyte activation in the marine rock shrimp *Sicyonia ingentis* takes place upon contact with seawater and is independent of fertilization (Pillai and Clark, 1987). The appearance of a primary extracellular coat around the "egg" is usually associated with activation of the oocyte. The components of this extracellular, or extra-embryonic, coat determine, in part, the resistance of the embryo to invasion by pathogens and establish a microenvironment for the developing zygote. In *S. ingentis* eggs, this extracellular coat appears about 45 min after spawning (Pillai and Clark, 1987, 1988). The presence of carbohydrate linkages in the extracellular coat of the crustacean egg is suggested by the abundance of saccharides in hatching envelopes (HE) isolated from shrimp (Pillai and Clark, 1990), by lectin labeling in shrimp HE (Pillai and Clark, 1990), and by the localization of *N*-acetylglucosamine in the copepod fertilization envelope (Santella, 1993). Pillai and Clark (1990) demonstrated carbohydrate components, in particular, *N*-acetylglucosamine and mannose, in *Sicyonia ingentis* HE.

We postulate that at least three enzymes are necessary for assembly of the HE in penaeoid shrimp eggs: an oxidase (Glas *et al.*, 1995), a transaminase (Glas *et al.*, 1992), and a carbohydrate synthase (Glas *et al.*, 1993). One of the most abundant carbohydrate components of decapod crustaceans that require a carbohydrate synthase is the polymer of β-1,4-*N*-acetylglucosamine, more commonly known as chitin. In this work, we show that enzymes that hydrolyze chitin or inhibit chitin synthase change HE elevation and assembly. In addition, chitin formation is reported to be dependent on ion concentration and composition (Warner, 1977; Stevenson, 1985). Clark and Lynn (1977) and Pillai and Clark (1987) reported that calcium and magnesium ions are necessary for the initial elevation of penaeid egg HE. In this study, we used ion-deficient seawater to determine the longer term effects of divalent ions on HE assembly and elevation in *S. ingentis* eggs. These morphological changes

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Abbreviations: hatching envelope, HE; ethylenediaminetetraacetic acid, EDTA; ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid, EGTA; transmission electron microscopy, TEM; fluorescein isothiocyanate, FITC; tetramethylrhodamine isothiocyanate, TRITC.

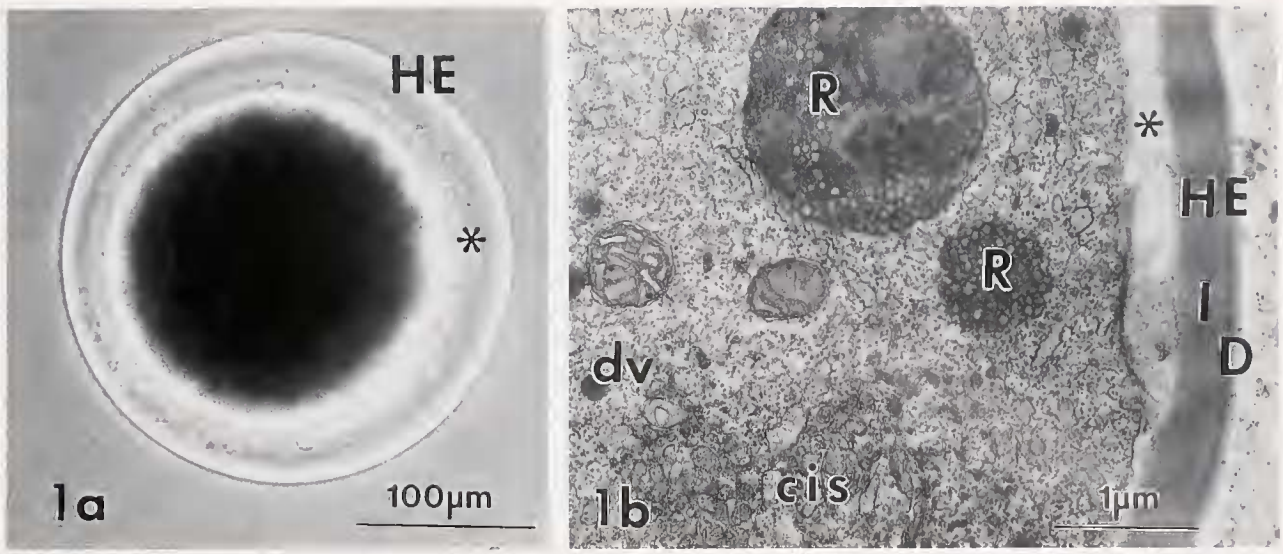


Figure 1. Eggs of *Sicyonia ingentis* 90 min postspawn in artificial seawater. (a) Eggs spawned into artificial seawater elevated a hatching envelope (HE) with a distinct pervitelline space containing a refractile material. Bar equals 100 μm. (b) Eggs spawned into seawater elevated a bilayered HE with a dense outer layer and a less dense inner layer. Bar equals 1 μm. HE, hatching envelope; D, dense layer; I, inner layer; *, perivitelline space; R, ring vesicles; dv, dense vesicles; cis, endoplasmic reticulum cisternae.

offer evidence of a chitin-like carbohydrate linkage in penaeid HE.

Materials and Methods

Gamete collection and handling

The rock shrimp *Sicyonia ingentis* was collected by otter trawl from waters off the southern coast of California adjacent to Santa Barbara and Los Angeles. Animals were transported in cooled, aerated tanks to Bodega Marine Laboratory, Bodega Bay, California, where they were maintained with constant light in flow-through aquaria. Spawning was induced by placing the females in darkness according to the methods of Pillai *et al.* (1988). Spawning females were held over 50 × 70 mm crystalizing dishes filled with artificial seawater at room temperature (20°C) to collect the eggs. Artificial seawater was prepared according to Cavanaugh (1956), with salinity adjusted to 30‰. Controls for each experiment were maintained in the artificial seawater at room temperature (~22°C) during the experiments.

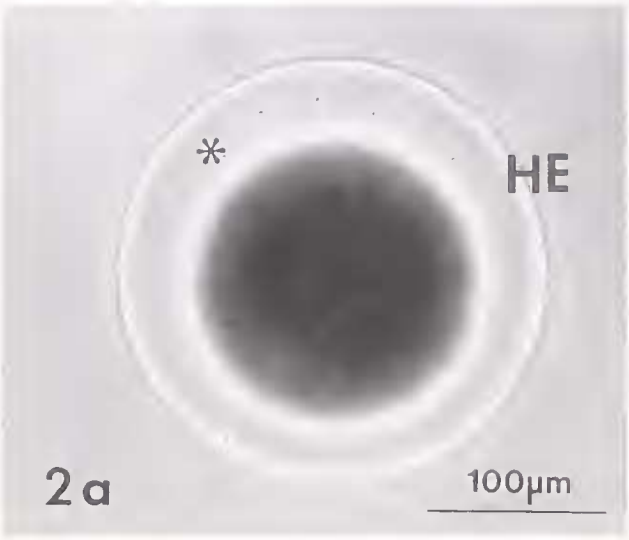
Inhibitors of fungal chitin synthesis, tunicamycin, nikkomycin Z, and polyoxin D (Calbiochem), were added to the eggs in seawater (pH 8.0) to give a final concentration of 1 μ*M*. Chitin hydrolytic enzymes, chitinase (1 μg/ml or 1.5×10^{-4} units) (Sigma, C-1525) and *N*-acetylglucosaminidase (0.05 units/ml) (Sigma, A-3189), were added to eggs in seawater at 10 min postspawn. Samples were removed for microscopic examination at 90 min postspawn—*i.e.*, well after normal formation of the HE, a fertilization-independent event (Pillai and Clark, 1987).

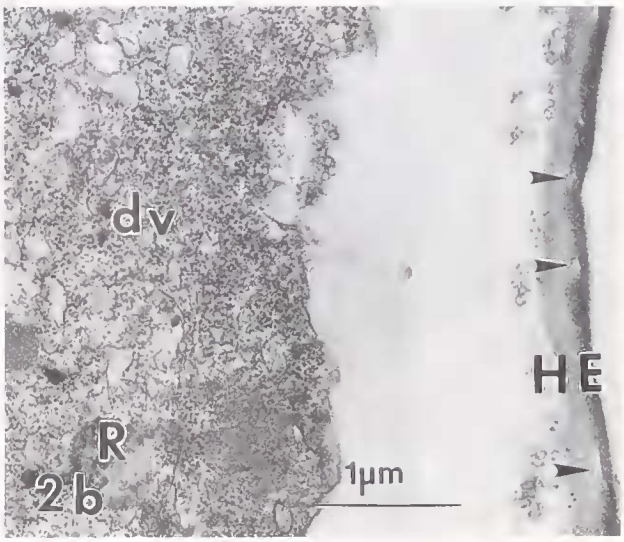
To observe the effects of ion dependency on HE assembly, seawaters deficient in calcium ions, magnesium ions, or both were used. Chelator concentrations of 5 m*M* EDTA (for calcium and magnesium) or 2 m*M* EGTA (for calcium) were added to the ion-deficient seawater to bind extraneous calcium and magnesium ions. The seawater was adjusted with sodium salts to maintain osmolarity. For ion-deficiency experiments, eggs were spawned into artificial seawater, and after 10 min the water was changed three times with the appropriate ion-deficient seawater. Thus, interference with early, ion-dependent events in egg activation was avoided (Clark and Lynn, 1977; Lindsay *et al.*, 1992).

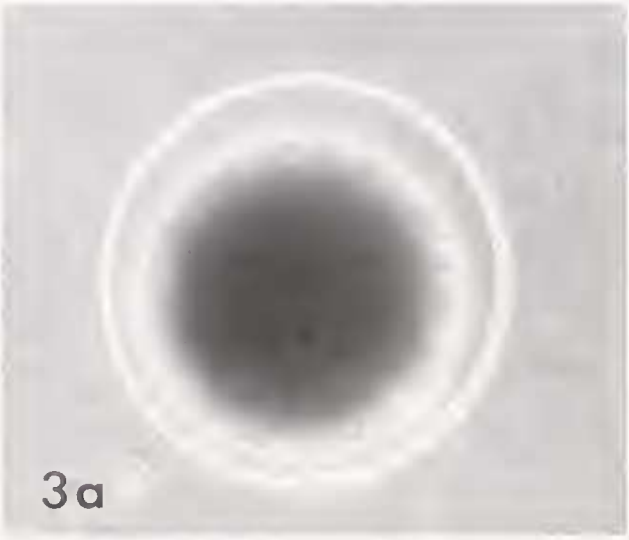
Microscopy

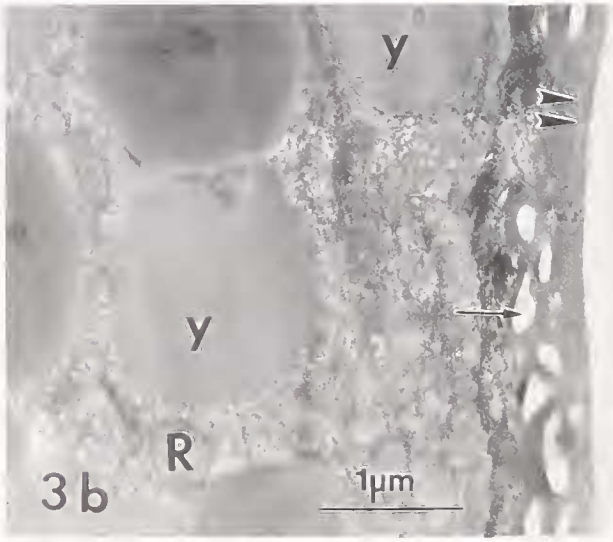
Aliquots of eggs from the spawning dish were placed on clean glass slides with coverslips supported by silicon grease to prevent desiccation and mechanical disruption of the eggs. Fresh aliquots were periodically prepared from the appropriate spawning dishes. Eggs were observed and photographed with phase microscopy during each experiment.

Samples for histology and electron microscopy were removed at 90 min postspawn and fixed with 1.2% para-


*
HE
2a
100μm


dv
HE
R
2b
1μm


3a


y
y
R
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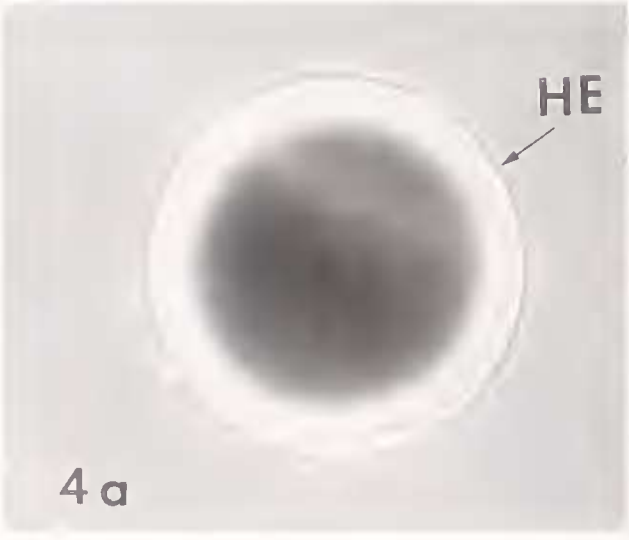
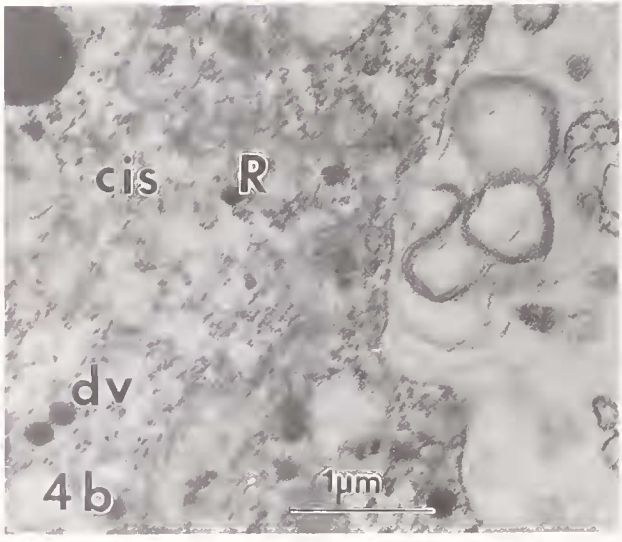

HE
4a


cis
R
dv
4b
1μm

formaldehyde and 0.8% glutaraldehyde in 0.28 *M* sodium cacodylate buffer in 33% seawater, pH 8.3, for 1 h at room temperature.

Electron microscopy

Samples for transmission electron microscopy (TEM) were washed three times in 0.4 *M* cacodylate buffer and post-fixed with a final concentration of 1% osmium tetroxide in 0.2 *M* cacodylate buffer for 45 min at room temperature. Samples were then rinsed, dehydrated through a graded acetone series, and embedded in a modified Spurr's resin (Spurr, 1969), substituting Quetol 651 for DER 736 resin. Thin sections were cut with a diamond knife, floated on copper grids, double stained with lead citrate (Venable and Coggeshall, 1965) and methanolic uranyl acetate, and viewed by TEM.

Hatching envelope isolation and lectin binding

Hatching envelopes from *S. ingentis* eggs 90 min postspawn were isolated according to the method of Pillai and Clark (1990) and Wikramanyake and Clark (1994). The eggs were homogenized in a Potter-Elvehjem homogenizer in 0.5 *M* NaCl with 5 m*M* benzamidine hydrochloride and 0.1% Nonidet (Sigma Chemical Co.). For the homogenization, the solution was dropped to pH 5 with a few drops of HCl to help with removal of the lipid. Subsequent washes were at pH 7.8. The homogenate was diluted 10 times with the homogenizing solution and centrifuged at 200 × *g* for 5 min. The pellet was washed repeatedly until translucent, then stored in liquid nitrogen.

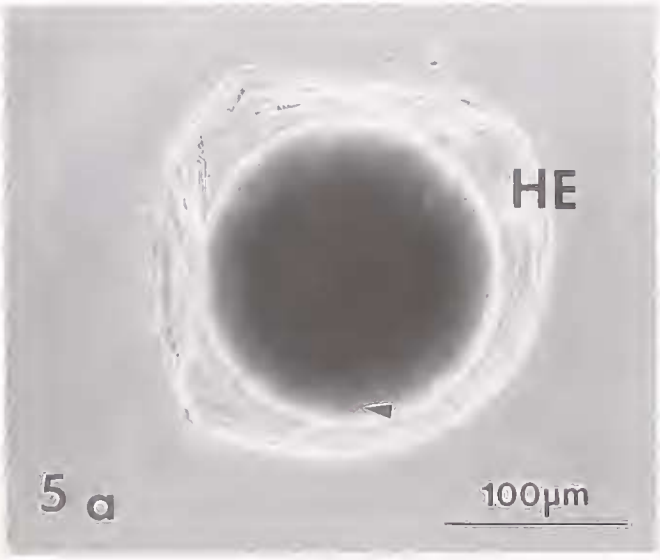
The frozen samples were thawed and lectin-binding assays on envelopes from the same isolation procedure were carried out followed the methods of Kiernan (1990) and Pillai and Clark (1990). Lectins labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC), both at 50 μg/ml, were used to identify terminal sugars. Concanavalin A (Con A) for mannose and glucose, wheat germ agglutinin (WGA) for *N*-acetylglucosamine and sialic acid, *Lens culinaris* agglutinin (LCA) for mannose, and *Bandeirea simplicifolia* (BS-II) for *N*-acetylglucosamine (all lectins from Sigma Chemicals) were applied in a 0.1 *M* phosphate buffer, pH 7.2, with trace metals added. Control samples contained 200 m*M* solutions of the appropriate blocking sugars (mannose, glucose, and *N*-acetylglucosamine) preincubated with the lectin for 10 min prior to application to the section (Kiernan, 1990; Pillai and Clark, 1990). Isolated HEs were incubated in the presence of the fluorescently labeled lectins for 1 h, then rinsed three times in phosphate buffer. Samples were observed with an epifluorescent microscope equipped with dichroic filter blocks for FITC and TRITC.

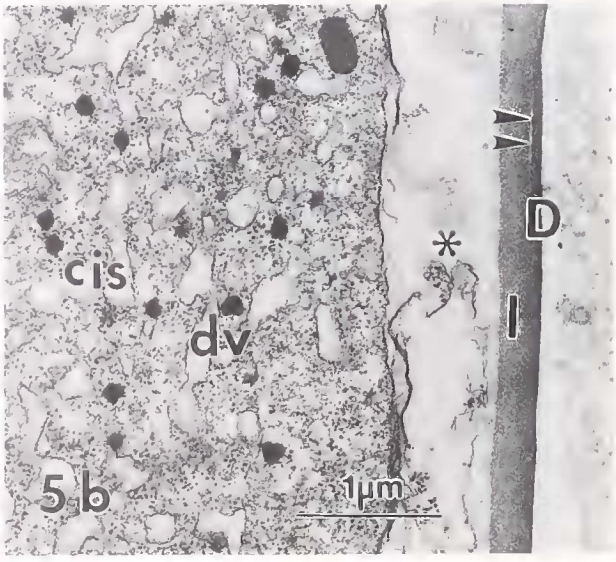
Results

Spawned eggs were observed for normal HE assembly and elevation in the presence or absence of chitin synthase inhibitors, chitin hydrolytic enzymes, and divalent ions. Criteria for normal elevation were the continued separation of the HE from the oolemma, as viewed by light microscopy, and the presence of the bilayered structure indicative of normal HE assembly, as described by Pillai and Clark (1988).

The control eggs began elevating the HE 40–50 min postspawn and showed normal HE formation at 60 min postspawn (Pillai and Clark, 1987, 1988). By 90 min postspawn, a refractile HE was formed around the egg (Fig. 1a). The perivitelline space contained a refractile material that is believed to add to the HE until the next extra-embryonic envelope is formed. Viewed with TEM, the HE had a thin, electron-dense outer layer and a thicker, less electron-dense inner layer (Fig. 1b) formed initially from components of the ring vesicles, as described by Pillai and Clark (1988). The cortex of the egg still contained many small, electron-dense vesicles and

Figures 2–4. Eggs of *Sicyonia ingentis* treated with chitin synthase inhibitors elevate an abnormal hatching envelope (HE). (2a) Eggs treated with tunicamycin sometimes appeared normal, with an elevated HE. The material in the perivitelline space looked finer than in the control eggs. Bar equals 100 μm. (2b) When viewed with TEM, the HE around eggs treated with tunicamycin appeared similar to the control eggs, but there are "holes" between the HE layers (arrowhead). The micrograph is in two sections to allow a greater view of the cortex of the egg. Bar equals 1 μm. (3a) Eggs treated with polyoxin D elevated envelopes that appeared to have a normal HE. (3b) When viewed with TEM, eggs in polyoxin D had abnormal HEs with clear spaces or "holes" in the inner layer (arrow) and between the two envelope layers (arrowhead). Large yolk granules (y) displaced the ring vesicles and mitochondria to the cortex of the egg. (4a) Eggs treated with nikkomycin Z elevated HEs (arrowhead) that remained close to the egg. (4b) Eggs in nikkomycin Z viewed with TEM appeared to have lost the HEs in the fixation and embedding process. The cisternae and dense vesicles were situated normally, but the ring vesicles (R) were not as packed as in the controls. HE, hatching envelope; D, dense layer; I, inner layer; *, perivitelline space; R, ring vesicles; dv, dense vesicles; cis, endoplasmic reticulum cisternae; y, yolk platelets.


HE
5 a
100µm


cis
dv
*
D
I
5 b
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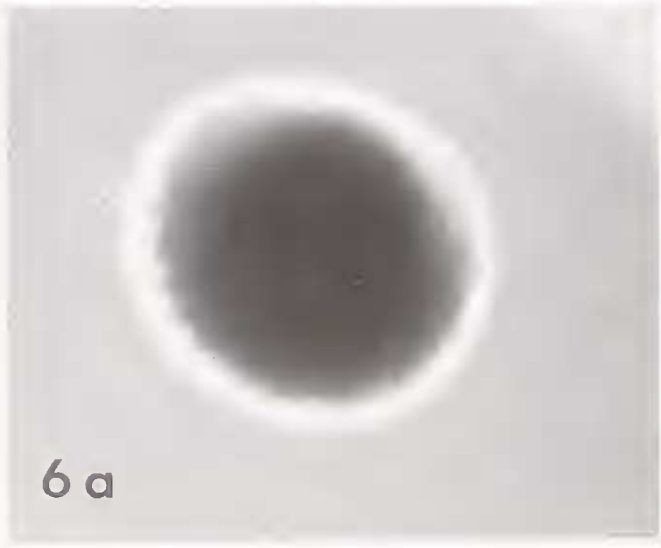
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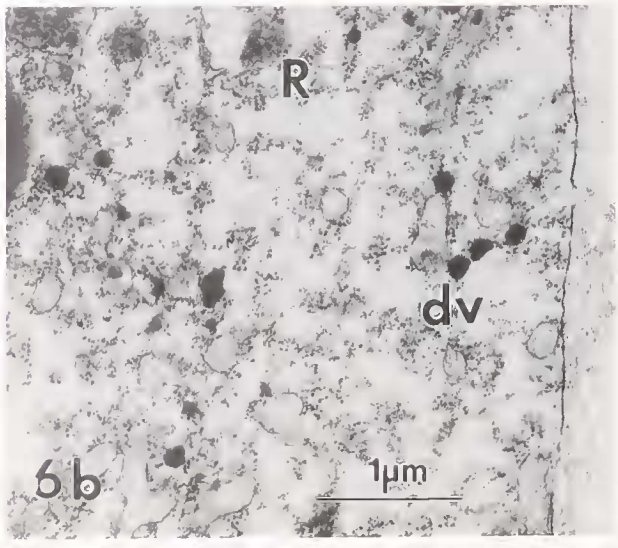
R
dv
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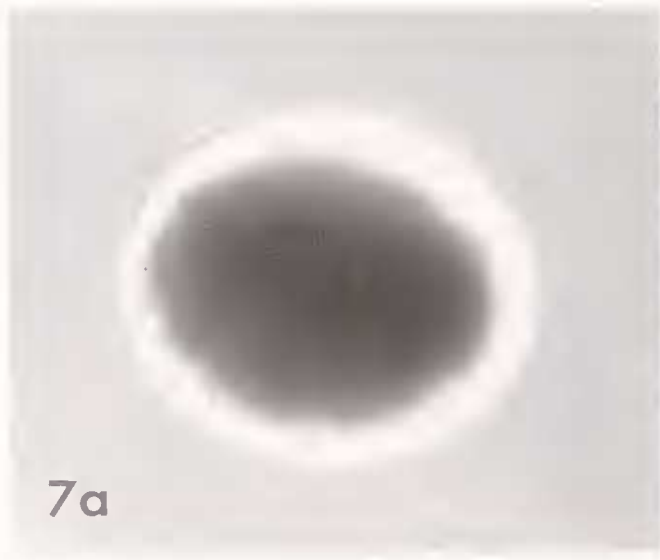
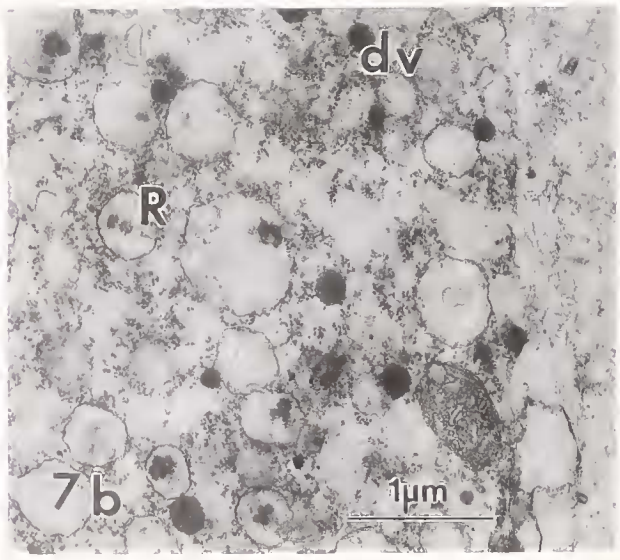

7a


dv
R
7 b
1µm

large ring vesicles tightly packed with ring-shaped elements. These vesicles appeared similar to those observed by Pillai and Clark (1988) during the early formation of the HE. Small cisternae of endoplasmic reticulum (ER) occupied much of the cortical region (Fig. 1b). Vesicles loosely filled with ring elements and mitochondria in close association with Golgi complexes were frequently observed. For a more detailed description of early HE formation, refer to Pillai and Clark (1988, 1990).

Chitin synthesis inhibitors block normal HE formation

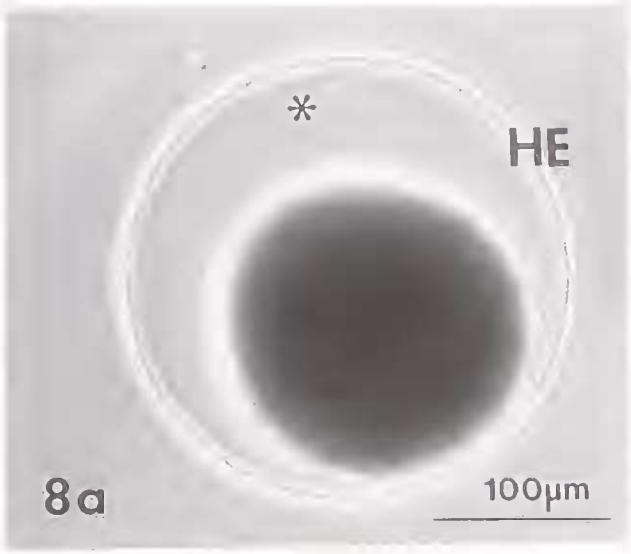
Eggs were treated with three chitin synthase inhibitors, tunicamycin, nikkomycin Z, and polyoxin D. In the treated eggs, HE formation, elevation, or both were abnormal. Eggs treated with tunicamycin elevated HE in two out of three trials when assayed with light microscopy (Fig. 2a). In some eggs, the HE remained close to the egg surface so that the perivitelline space was obscured (not shown). Granular material finer than that observed in control eggs was seen in the perivitelline space. Eggs treated with polyoxin D had envelopes that appeared similar in structure to the control egg when viewed with light microscopy (Fig. 3a). In eggs treated with nikkomycin Z, the HE remained close to the egg surface (Fig. 4a) and the perivitelline space was filled with a fine material, often making it difficult to visualize the separation from the egg surface when viewed with light microscopy.

Eggs treated with tunicamycin and examined with TEM had a bilayer HE as in control samples, but the two layers had electron-translucent areas, or "holes," between the thin electron-dense outer layer and the flocculent inner layer (Fig. 2b). This condition resembled normal eggs during early elevation (*i.e.*, 60 min postspawn), suggesting a delay in HE formation (see Pillai and Clark, 1988, for comparison). Incorporation of exocytosed ring vesicles into the HE was incomplete, and the ring elements remained conspicuous in the perivitelline space. The ER cisternae and the mitochondria were less conspicuous in the peripheral cortex (Fig. 2b) compared to control eggs. In TEM preparations of eggs treated with polyoxin D, the HE was abnormal and collapsed to the oolemma surface (Fig. 3b). The flocculent inner layer was interrupted by many large electron-translucent areas (holes). The outer layer and inner layer had a series of fine electron-translucent spaces separating the layers. The cortex of eggs treated in polyoxin D, unlike that of control eggs, was filled with prominent yolk platelets. The peripheral relocation of the yolk appeared to compress the ER and mitochondria in the egg cortex. Small electron-dense vesicles were not seen in the peripheral cortex. In TEM micrographs, eggs treated with nikkomycin Z lost the HE during processing (Fig. 4b), indicating an extremely fragile HE. Numerous vesicular structures were adjacent to the egg surface. The cortical region resembled that of an egg during early HE formation and elevation, with many small electron-dense vesicles and large vesicles containing a few ring elements.

Chitin hydrolytic enzymes inhibit normal envelope assembly

Sicyonia ingentis eggs were treated with the chitin hydrolytic enzymes (chitinase and *N*-acetylglucosaminidase) at 10 min postspawn and observed until 90 min postspawn. Chitinase-treated eggs formed HEs that initially elevated farther from the eggs than in the controls, then collapsed (Fig. 5a). Hatching envelopes were not apparent in *N*-acetylglucosaminidase-treated eggs in three out of three trials (Fig. 6a). When both *N*-acetylglucosaminidase and chitinase were added to the spawning media, again no HEs were discernible around the eggs (Fig. 7a),

Figures 5–7. Eggs of *Sicyonia ingentis* treated with chitinolytic enzymes do not elevate a normal hatching envelope (HE). (5a) Eggs treated with chitinase elevated HEs, but the envelopes appeared thinner than the control envelopes and usually collapsed to the oolemma surface. Bar equals 100 μm. (5b) The HE of eggs treated with chitinase appeared to have formed with the two layers, but the inner layer was not as thick as in control eggs. The dense layer (D) was separated from the inner layer (I) by small holes (arrowhead). The perivitelline space (*) contained finer material than seen in the control eggs. Endoplasmic reticulum cisternae and dense vesicles filled the egg cortex, but no packed ring vesicles were seen near the cortex. Bar equals 1 μm. (6a) Eggs treated with *N*-acetylglucosaminidase did not have elevated HEs. (6b) *N*-acetylglucosaminidase-treated eggs did not form normal HEs. Dense vesicles were seen in the cortex, and the packed ring vesicles were present, but the integrity of the cortical region was disrupted. Bar equals 1 μm. (7a) Eggs treated with both chitinase and *N*-acetylglucosaminidase did not elevate normal HEs. The eggs appeared swollen with a highly refractive cortex. (7b) Eggs treated with both chitinase and *N*-acetylglucosaminidase did not have HEs when viewed with TEM. The exterior of the oolemma resembled the surface of an egg in early HE formation. The large ring vesicles had only a few ring elements in them. Again, the structural integrity of the cortex appeared disrupted. Bar equals 1 μm. HE, hatching envelope; D, dense layer; I, inner layer; *, perivitelline space; R, ring vesicles; dv, dense vesicles; cis, endoplasmic reticulum cisternae; y, yolk platelets.


*
HE
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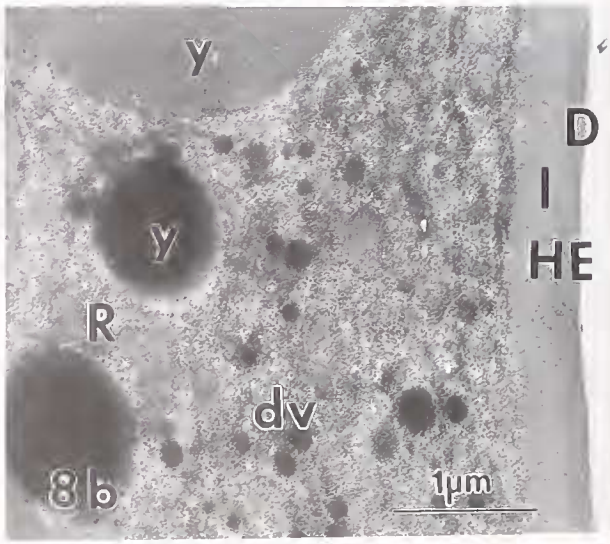
y
D
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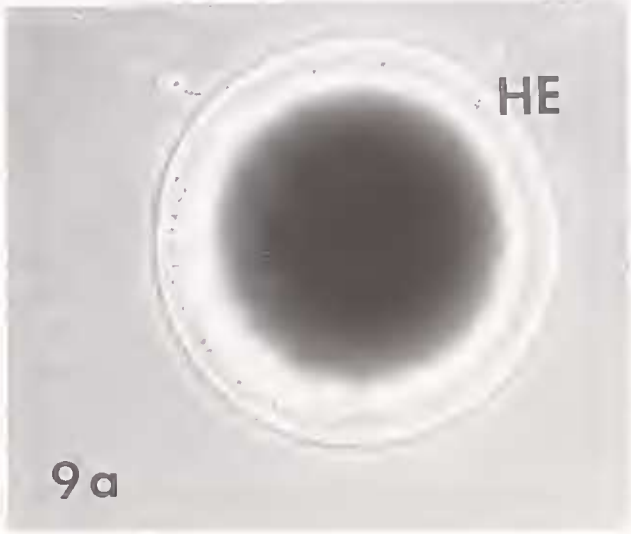
HE
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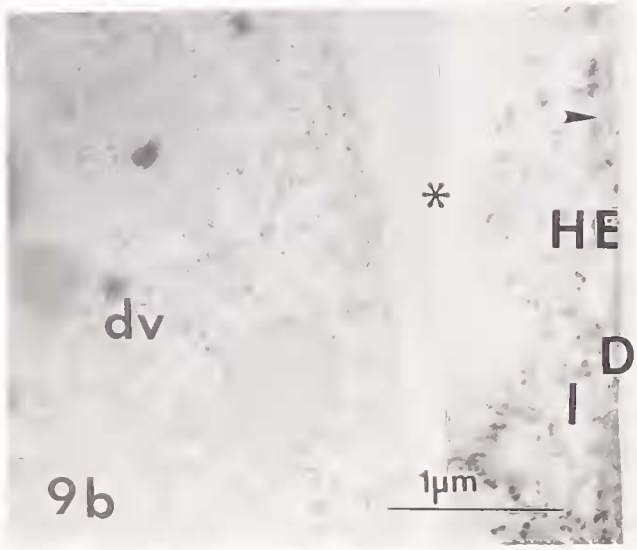
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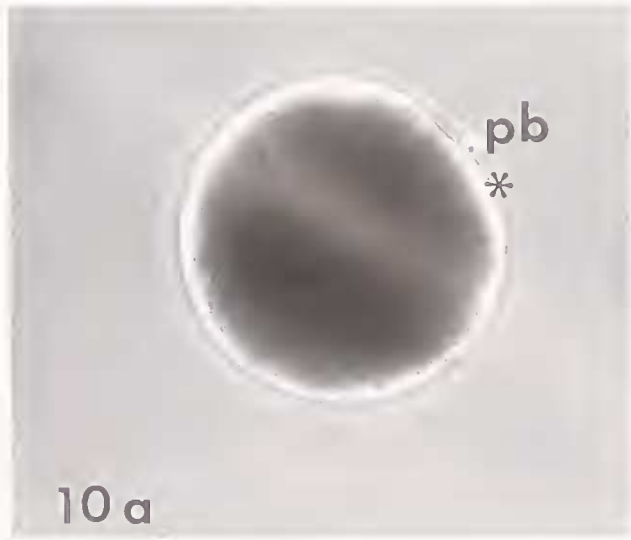
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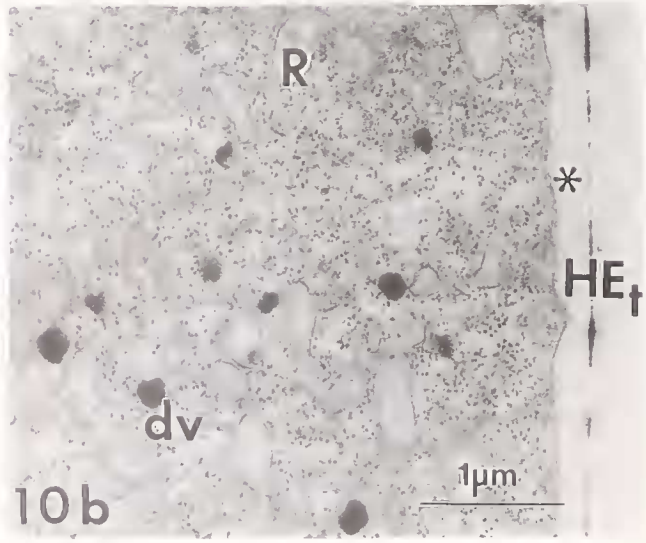
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and the egg appearance resembled that of a newly spawned egg (see Pillai *et al.*, 1988). These eggs often appeared swollen, with a highly refractive cortex. Eggs in *N*-acetylglucosaminidase alone or both chitinase and *N*-acetylglucosaminidase were very fragile and broke easily when pipetted or swirled vigorously in the spawning dish.

With TEM, the HEs of the chitinase-treated eggs were bilayered, similar in appearance to control envelopes (Fig. 5b). The ER cisternae were as numerous as in control eggs, but appeared more electron-translucent, suggesting lack of material content. Small dense vesicles were abundant, but the large packed ring vesicles were not seen. TEM of *N*-acetylglucosaminidase-treated eggs showed a thin, unorganized layer of material resembling the HE precursor (see Pillai and Clark, 1988) on the exterior of the egg membrane (Fig. 6b). The eggs lacked structural integrity, suggesting eventual cell death. Vesicles with ring structures were smaller than in the control eggs. The small dense vesicles were similar to those in control eggs. When viewed with electron microscopy, eggs treated with a combination of both enzymes had a thin, unorganized area visible external to the oolemma (Fig. 7b); this area resembled the HE precursor. Dense vesicles were abundant in the cortex, and the large vesicles contained only a few ring elements. The leached appearance again suggested extreme disruption of cell function leading to cell death.

Ion deficient seawaters affect normal envelope assembly

In the following experiments, eggs were spawned in artificial seawater, washed three times in the appropriate ion-deficient seawater at 10 min postspawn, and allowed to develop. In calcium-deficient seawater, the HE, as observed with phase microscopy, elevated farther from the egg surface than in controls (Fig. 8a). The egg was usually eccentric within the HE, and the HE would collapse when eggs were handled. Elevation of HE by eggs in magnesium-deficient seawater appeared relatively normal (Fig. 9a), although the HE did not appear to elevate as far from the egg as in the controls. Granular material similar to that in control eggs was visible in the perivitelline space. No elevated HE were observed in eggs treated with seawater deficient in both calcium and magnesium, although a thin HE precursor could be seen covering the second polar body (Fig. 10a). The eggs were very fragile, breaking easily with handling.

Examination of eggs from calcium-deficient seawater with electron microscopy showed an HE with the bilayer morphology, but the envelopes had collapsed to the oolemma (Fig. 8b), again suggesting lack of tanning of the envelope. Small dense vesicles were abundant in the cortex of the egg, but the mitochondria and cisternae were misshapen by yolk platelets. Eggs in magnesium-deficient seawater showed HEs with a thin bilayered construction (Fig. 9b). However, the HE had large electron-translucent areas ("holes") between the two layers and within the inner layer. The ring elements did not appear to be incorporated into the HE. The ER cisternae were prominent in the cortex. Ninety min postspawn eggs treated with calcium- and magnesium-deficient seawater showed HEs that were morphologically similar to HEs during early formation (Fig. 10b) (see Pillai and Clark, 1988, for comparison). The ring vesicles were not as packed as in the controls, and the cortex appeared similar to that of a normal egg during the period immediately before normal HE elevation and assembly.

Shrimp-isolated HEs label with β-glucoside specific lectins

Envelopes isolated from eggs at 90 min postspawn were incubated with fluorescently labeled lectins to detect the presence of carbohydrates: Concanavalin A (Con A) for mannose and *N*-acetylglucosamine, wheat germ agglutinin (WGA) for *N*-acetylglucosamine, *Lens culinaris* agglutinin (LCA) for mannose, and *Bandeirea simplicifolia* (BS-II) for *N*-acetylglucosamine. Untreated iso-

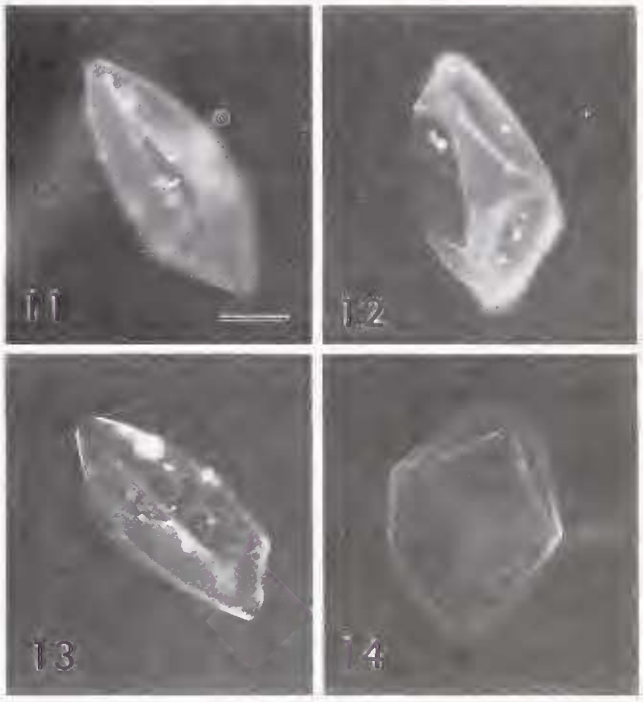
Figures 8–10. Eggs incubated in ion-deficient seawater do not elevate a normal hatching envelope (HE). (8a) Eggs treated with calcium-deficient seawater had an HE that appeared to elevate farther from the egg than in the controls. Bar equals 100 μm. (8b) Eggs treated in calcium-deficient seawater had an HE that was collapsed to the oolemma, although a bilayered structure could be seen. Yolk and lipid droplets were close to the oolemma. Bar equals 1 μm. (9a) In magnesium-deficient seawater, the HE did not elevate as far from the egg as in the controls. (9b) The HE in magnesium-deficient seawater was filled with electron-translucent areas, or "holes," between the two layers of the HE (arrowhead). The inner layer (I) was not as well organized as in the control or in calcium-free seawater. The micrograph is in two sections to allow a greater view of the cortex of the egg. Bar equals 1 μm. (10a) In calcium- and magnesium-deficient seawater, the HE did not elevate from the egg surface although a thin HE precursor could be seen covering the second polar body (pb). (10b) In calcium- and magnesium-deficient seawater, a normal HE was not formed. On the exterior of the oolemma was a thin layer, probably the HE precursor (HE_1), but the dense layer and inner layer had not formed. Bar equals 1 μm. HE, hatching envelope; D, dense layer; I, inner layer; *, perivitelline space; R, ring vesicles; dv, dense vesicles; cis, endoplasmic reticulum cisternae; y, yolk platelets.

lates showed no fluorescence (not shown). LCA and ConA strongly labeled the isolated HEs (Figs. 11 and 12). WGA (Fig. 13) and BS-II (Fig. 14) showed much weaker staining, and BS-II showed only a slight reaction with the isolated envelopes.

Discussion

The focus of this study is on the formation and elevation of the shrimp HE after exposure of the eggs to chitin hydrolytic enzymes or chitin synthesis inhibitors. However, the potential role of the cortex in the synthesis of any chitin-type carbohydrate cannot be ignored. Thus, the morphology of the cortex is an important aspect of HE formation. The cortical region of the penaeid shrimp egg at 90 min postspawn has the classic features of a biologically active cell—abundant mitochondria, endoplasmic reticulum, and cisternae. At least two types of vesicles with morphologically different characteristics are present, suggesting additional synthetic activity. Observations in this laboratory have shown that the HE continues to have material added to it until the next envelope is constructed at 90–110 min postspawn. Consequently, biochemical changes such as cross-linking (tanning or hardening) of the HE and incorporation of new material into the HE may continue until much later than originally expected and ultimately result in the appearance of chitin-like components in the HE. The three experimental approaches used in this study, *i.e.*, inhibitory, hydrolytic, and ionic treatments, resulted in visible changes in the morphology and assembly of the HE, as well as in the morphology of the cortex. These results were dramatically different from the normal sequence of events described in earlier investigations (Pillai and Clark, 1988, 1990; Lynn *et al.*, 1991, for review). Interestingly, the treatments often result in eggs that appear to have been arrested in earlier stages of development.

When eggs are treated with chitin synthase inhibitors, HE elevation occurs, but the structure of the HE and the morphology of the cortical region of the egg are affected by the synthase inhibition. The collapse or loss of the HE during handling suggests extreme fragility or susceptibility to chemical stress. The severity of the morphological abnormalities differs with the actions of the chitin synthase inhibitors. This is not surprising because the inhibitors are known to react differently between the insects and other crustaceans (Cabib, 1987, 1991; Cohen, 1987). Tunicamycin is an antibiotic that blocks the formation of protein-carbohydrate linkages of the *N*-glycosidic type (Duskin and Mahoney, 1979). Polyoxin D is a specific inhibitor of fungal chitin synthase that is known to result in malformed endocuticular layers in the larvae of the butterfly, *Pieris brassicae*, a cabbage pest (Cohen, 1987).



Figures 11–14. Fluorescent micrographs show labeling of isolated hatching envelopes (HEs) from *Sicyonia ingentis* eggs at 90 min postspawn. Labeling as follows: (11) LCA lectin; (12) ConA lectin; (13) WGA lectin; (14) BS-II lectin. Note the relative decrease in labeling for HEs treated with WGA and BS-II. Untreated control HEs showed no background fluorescence. Bar equals 50 μm.

Nikkomycin Z is a competitive inhibitor of chitin synthase in fungal cell walls (Cabib, 1987). Although these antibiotics are reported to inhibit chitin synthetic pathways, the possibility of action on other related or unrelated metabolic pathways in decapod development cannot be dismissed.

Treatment of the eggs with two enzymes that are considered to be part of the chitinolytic pathway results, again, in abnormal elevation and assembly of the HE. With chitinase treatment an HE is elevated and appears somewhat normal in structure, but the collapse of the HE suggests abnormal hardening (*i.e.*, cross-linking) of the envelope. Although preliminary assays indicated some proteolytic activity in the chitinase enzyme, proteolytic inhibitors could not be simultaneously used because of their deleterious effect on HE elevation and morphology. In addition, the chitinase results indicate relatively subtle changes in HE formation in contrast to what occurs in proteolytic treatments of the HE. Thus while proteolytic effects cannot be ruled out, the results of the chitinase treatment are not consistent with such effects. Eggs treated with *N*-acetylglucosaminidase, the chitinolytic enzyme, appeared not to elevate an HE. The

chitinolytic enzymes do not appear to degrade the HE from the outside, as might be expected; instead, they seem to affect the assembly *per se*. Since the pH optima for chitinase and *N*-acetylglucosaminidase are 6 and 4, respectively (Muzzarelli, 1977), hydrolytic activity is unlikely to occur at the pH of seawater (pH 8.0). Thus, these enzymes are probably binding to substrate and effectively blocking further polymerization. The leached appearance of the cortex of eggs treated with *N*-acetylglucosaminidase suggests that one mode of action may be the disruption of the cortical architecture. Whether this disruption interferes directly with the release of a necessary substrate or enzyme or indirectly through cell death is unclear. However, the assembly of the HE in the presence of chitinase alone suggests that the *N*-acetylglucosaminidase is necessary for the lack of HE formation, much as the combination of enzymes is necessary for complete degradation of chitin.

Changes in ionic composition of the seawater after initial egg-sperm interaction has occurred also affect the assembly of the HE. Deficiency of Mg^{+2} and Ca^{+2} ions results in eggs with abnormal HE formation, cortical composition, or both (Clark and Lynn, 1977; Pillai and Clark, 1987). In the eggs treated with ion-deficient seawater, the holes between the two HE layers and within the inner layer suggest lack of incorporation of materials into the HE. In particular, the packed ring vesicles seem to be affected by these ionic deficiencies, as treated eggs were lacking the large number of packed ring vesicles found in the control eggs. The morphology of ion-depleted eggs suggests an interruption or delay in HE formation. As suggested for early egg activation (Clark and Lynn, 1977; Pillai and Clark, 1987), Mg^{2+} appears to be more important than Ca^{2+} for normal envelope assembly. The presence of at least one of these ions is necessary for normal HE assembly.

The lectin study showed some differences from results reported by Pillai and Clark (1990). However, the difference in the time points of the samples may account for some of the discrepancies. The results suggest that although glucose and mannose residues are present in the HE, labeling for *N*-acetylglucosamine oligomers by BS-II and WGA suggested either less accessibility or less abundance compared to the results of Pillai and Clark (1990).

The results of this study, together with previous demonstration of an oxidase (Glas *et al.*, 1995), offer argument for the presence of a chitin-like, or similarly linked, carbohydrate component in the HE of penaeid shrimp. The morphology of the penaeid egg during early development has all the characteristics of a biochemically active cell. The construction of the HE and the extra-embryonic envelopes that are formed later is a dynamic function of this cell. Further exploration of the enzymes and resulting events during this period could offer new insights into the mechanics of embryo protection and activity during early development.

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